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Effects of Temperature, Amebic Strain, and Carbohydrates on *Acanthamoeba* Adherence to Corneal Epithelium In Vitro

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An in vitro coincubation assay was used to measure adhesion of radiolabeled *Acanthamoeba* trophozoites to corneal epithelium. Adhesion of amebae to corneal epithelium was higher at 25°C than at 37 or 4°C, did not consistently correlate with the reported pathogenicity of the strain of *Acanthamoeba*, and was inhibited by mannose and by *methyl*-α-D-mannopyranoside.

Acanthamoeba keratitis is an increasingly reported and often unmanageable protozoal infection occurring primarily in wearers of soft contact lenses. Little is known about the pathogenesis of this disease or about what combination of host and parasite factors is necessary to initiate an infection.

Adherence of microorganisms to the cornea is a prerequisite for colonization and invasion of the corneal epithelium (4). Recognition of host cell glycolipids and/or glycoproteins by a variety of microbial pathogens appears to be significant in adherence of organisms to their target tissues and in determination of tissue tropism, species specificity, and genetic specificity of pathogens (2).

Pseudomonas aeruginosa, the most extensively studied contact lens-associated pathogen, has been demonstrated, both in vivo and in vitro, to adhere to the corneal epithelium at least partially via bacterial adhesins (pili) which recognize galactose, mannose, and sialic acid residues on the corneal surface and in contact lens deposits (1, 3, 9).

Entamoeba histolytica binds to its target tissue (colonic mucosa) by a surface lectin which recognizes galactose or *N*-acetyl-D-galactosamine residues in the colonic mucus or on the colonic epithelial cell (19). *E. histolytica*-induced cytolysis is a contact-mediated event which can be inhibited by the presence of competing galactose or *N*-acetyl-D-galactosamine. Adherence does not seem to be the sole virulence factor for *Entamoeba* strains. Nonpathogenic strains express the lectin as well as pathogenic strains and are equally adherent (18).

This paper describes the use of an in vitro adherence assay to characterize conditions for *Acanthamoeba* adherence to corneal epithelium, to determine whether strains of *Acanthamoeba* which differ in their in vivo virulence also differ in their in vitro capacity to adhere to corneal epithelium, and to determine whether recognition of carbohydrate moieties on the surface of the corneal epithelial cells may play a role in *Acanthamoeba* adherence to its target tissue.

Axenic trophozoites of *Acanthamoeba polyphaga*, *A. culbertsoni* Lilly A-1, *A. castellanii* 76/2252, *Acanthamoeba* sp. strain Neff, and *Acanthamoeba* sp. strain Mc were obtained from G. S. Visvesvara (Centers for Disease Control, Atlanta, Ga.). These five strains have been identified as genetically distinct on the basis of restriction fragment length characteristics (14). *A. polyphaga*, *A. culbertsoni* Lilly A-1, and *Acanthamoeba* sp. strain Mc are isolates from clinical cases of *Acanthamoeba* keratitis and are considered pathogens on the basis of mouse inoculation tests. *A. castellanii* 76/2252 and *Acanthamoeba* sp. strain Neff are environmental isolates and are considered nonpathogens on the basis of the same criterion. All *Acanthamoeba* strains were grown axenically at 25°C in 75-cm^2 plastic flasks (Falcon, Becton Dickinson & Co., Lincoln Park, N.J.) in peptone-yeast extract-glucose (PYG) medium (24). For adherence assays, trophozoites of *A. polyphaga*, *A. culbertsoni* Lilly A-1, and *A. castellanii* 76/2252 or of *A. polyphaga*, *Acanthamoeba* sp. strain Mc, and *Acanthamoeba* sp. strain Neff (two pathogenic strains and one nonpathogenic strain per group) were grown for 48 h prior to use. The trophozoites were collected by briefly chilling the flask and scraping the loosely adherent ameba from the flask surface with a sterile plastic cell scraper. Amebae were counted with a hemacytometer, and organisms from each experimental strain were resuspended for radiolabeling in 100 μl of fresh PYG medium. The amebae were pulse-labeled with 45 μCi of [^{35}S]methionine (specific activity, 49.3 TBq/mmol; Amersham Corp., Arlington Heights, Ill.) for 45 min at 25°C, with agitation every 15 min. The labeled amebae were washed three times with fresh PYG and resuspended in 50 ml of fresh PYG to a final concentration of 1×10^5 (for adhesion assays examining the effects of time, temperature, and strain) or 4×10^5 (for sugar inhibition studies) per ml.

Corneal epithelial cells were grown in tissue culture by using eyes from adult albino rabbits euthanatized by other researchers in the course of their own research or by laboratory animal care veterinarians for humane reasons. The eyes were removed intact. Each cornea was removed within the limbus, and the endothelium and posterior stroma were peeled from the posterior surface by using fine forceps and discarded. The remaining sheets of epithelium and anterior stroma were then cut into 2- to 3-mm^2 explants, placed epithelial side up in 100-mm plastic tissue culture dishes (Falcon 3808 Primaria; Becton Dickinson & Co.), air dried for 10 to 15 min, and then covered with primary culture medium consisting of equal parts of Hams F-12 and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Dulbecco's Modified Eagle Medium (GIBCO Laboratories, Grand Island, N.Y.) enriched with 5% Nu-Serum (Collaborative Research Inc., Bedford Park, Mass.), 50 μg of Gentacin (GIBCO) per ml, 5.0 μg of insulin (Collaborative Research) per ml, and 10 ng of epidermal growth factor (Collaborative Research) per ml (11). The dishes were incubated at 37°C in a humidified incubator with 5% CO_2. Epithelial outgrowth from the explant was usually apparent within 72 h. The medium was changed three times weekly.

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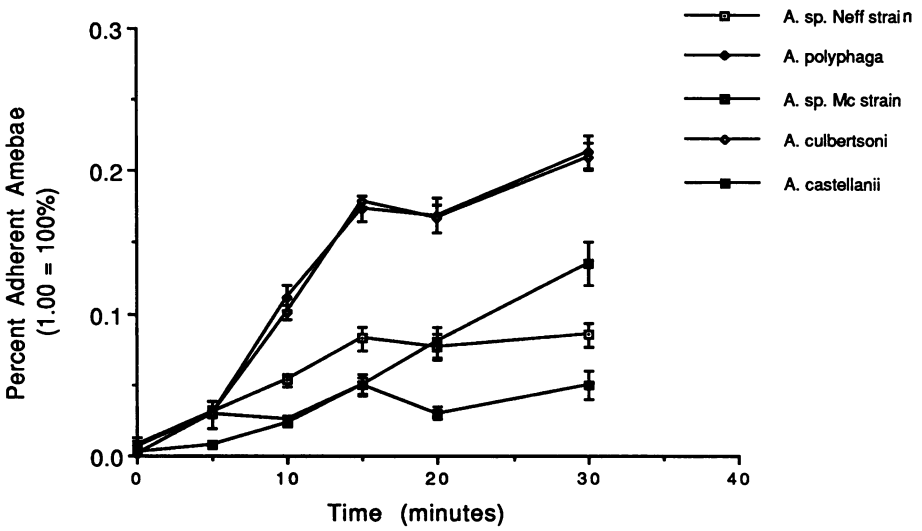

FIG. 1. Adherence of *Acanthamoeba* sp. strain Neff, *A. polyphaga*, *Acanthamoeba* sp. strain Mc, *A. culbertsoni* Lilly A-1, and *A. castellanii* 76/2252 to corneal epithelium in vitro at 25°C. Each point represents the mean of three experiments. Bars indicate standard error.

The epithelial nature of the cultured cells was confirmed by plating cells into tissue culture chamber slides (Lab-Tek, Miles Scientific, Naperville, Ill.) and staining for cytokeratin by the peroxidase-antiperoxidase method (22).

Once the outgrowth areas began to coalesce and cover the dish surface, the explants were transferred sterilely to new culture dishes for continued growth. The cellular outgrowth was detached from the culture plate by trypsinization and suspended in fresh growth medium. The cell concentration was determined with a hemacytometer and adjusted to 10^5/ml. Cell viability was >90%, as determined by trypan blue exclusion. Cell suspensions (10^5/ml) were seeded in wells of flat-bottom multiwell tissue culture plates (Falcon 3847 Primaria; Becton Dickinson & Co.) and incubated under 5% CO_2 in a 37°C incubator until confluent monolayers were formed (48 to 72 h). The culture medium was changed every 2 days. Immediately prior to use in adherence assays, the culture medium was removed and replaced by 0.5 ml of fresh PYG medium.

Adherence studies were performed by coincubating 5×10^4 radiolabeled trophozoites of *A. polyphaga*, *A. culbertsoni* Lilly A-1, and *A. castellanii* 76/2252 on epithelial monolayers for 5, 10, 15, 20, or 30 min at 4, 25, or 37°C. Trophozoites were also inoculated onto monolayers at 25°C and immediately washed off to establish adherence at 0 min. On the basis of results from initial studies, adherence of two additional strains of *Acanthamoeba* (*Acanthamoeba* sp. strain Mc and *Acanthamoeba* sp. strain Neff) was compared to adherence of *A. polyphaga* after 0, 5, 10, 15, 20, and 30 min at 25°C. At the end of the incubation period, the culture fluid was poured off the plates and the monolayers were washed twice with phosphate-buffered saline (PBS) to remove loosely adherent amebae. The monolayers were lysed with 1% sodium dodecyl sulfate (SDS), and the amount of ^{35}S activity in the lysate was determined with a beta counter (Packard Tri-Carb 460; Packard Instrument Co., Downers Grove, Ill.). Data were expressed as a percentage of the activity associated with a lysed aliquot containing 5×10^4 amebae (minimum of 62,000 cpm). All experiments were performed in triplicate.

Saccharides (sialic acid, galactosamine, *N*-acetylgalactosamine [GalNAc], glucosamine, *N*-acetylglucosamine [GlcNAc], galactose, mannose, *methyl*-α-D-mannopyranoside, and fucose) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled *A. polyphaga* trophozoites were suspended at 4×10^5/ml in PYG medium containing saccharides at 10 mM for 45 min before addition to the epithelial monolayers. The final pHs of all solutions, e.g., sialic acid, were adjusted to 6.5. A 500-μl aliquot of amebae-sugar suspension was mixed with 500 μl of PYG medium on the epithelial monolayers to yield a final sugar concentration of 5 mM and a final amebic concentration of 2×10^5 cells per ml. Amebae were coincubated with epithelial monolayers in the presence of competing sugar for 30 min at 25°C. Control assays using amebae suspended in PYG alone were conducted simultaneously. At the end of the incubation period, the medium was poured off and the monolayers were washed twice with PBS and lysed with 1% SDS for counting. Adherence in the presence of sugar was expressed as a percentage of adherence in PYG medium alone. Each comparison was performed in triplicate ($n = 36$), except for sialic acid ($n = 12$).

Differences between the experimental groups were determined by analysis of variance with StatView 512+ Graphics for the Macintosh (Abacus Concepts, Inc., Berkeley, Calif.). Differences were considered significant at the level of $P < 0.05$.

Maximal adherence for *A. polyphaga*, *A. culbertsoni* Lilly A-1, and *A. castellanii* 76/2252 occurred at 30 min of coincubation at 25°C. Adherence of *A. castellanii* 76/2252 was higher at 37°C for periods of less than 30 min but higher at 25°C for periods longer than 30 min (15). At 25°C (Fig. 1), adherence of these trophozoites to corneal epithelium increased steadily with time. At 4 and 37°C, adherence increased until 15 or 20 min of coincubation and then began to plateau and/or decline.

At all three experimental temperatures, adherence of the two pathogenic strains, *A. polyphaga* and *A. culbertsoni* Lilly A-1, was significantly greater than that of the nonpathogenic strain, *A. castellanii* ($P < 0.001$). However, additional assays comparing adherence at 25°C of *A. polyphaga* with adherence of a third pathogenic strain, *Acanthamoeba*

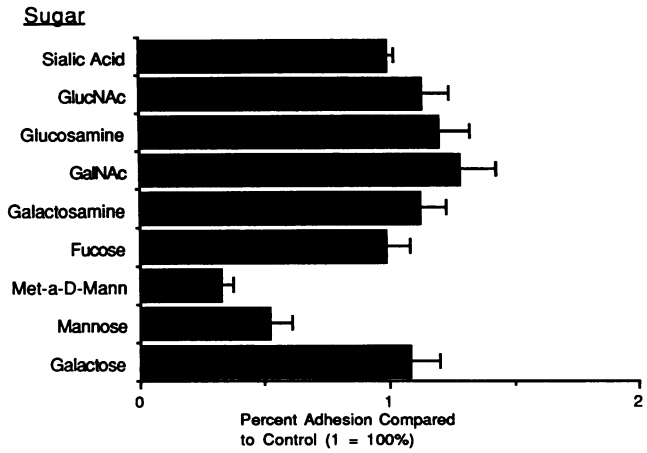

FIG. 2. Comparison of effects of saccharides on adherence of *A. polyphaga* to corneal epithelium in vitro. Each bar represents the mean of three experiments. Bars indicate standard error. Met-α-D-Mann, *methyl*-α-D-mannopyranoside.

sp. strain Mc, and that of a second nonpathogenic strain, *Acanthamoeba* sp. strain Neff, showed that although there were also significant differences ($P < 0.001$) in adherence of these strains, in this comparison the Neff strain was more adherent than the Mc strain (Fig. 1).

Adherence of *A. polyphaga* to corneal epithelial cells after 30 min at 25°C was significantly decreased by mannose (52.0% of control ± 8.9% [standard error]; $P = 0.0001$) and *methyl*-α-D-mannopyranoside (32.2% of control ± 5.1% [standard error]; $P = 0.0001$) (Fig. 2). Adherence was slightly augmented by glucosamine, GlcNAc, galactosamine, and GalNAc, but only the effect of GalNAc (128.1% ± 14.6% [standard error]) was significant ($P < 0.05$). Fucose, sialic acid, and galactose had no significant effect on amebic adherence.

A proposed pathogenesis of *Acanthamoeba* keratitis includes contamination of lens solutions by *Acanthamoeba* followed by exposure of the contact lens to contaminated solutions; adherence of cysts and trophozoites to the lens surface; persistence of cysts and trophozoites after inadequate cleaning and rinsing; introduction of *Acanthamoeba* organisms to the eye with the contact lens; entrance of cysts and trophozoites into the tear film; and amebic invasion of the corneal stroma either through intact epithelium or through breaks in the epithelium. Contact lens-induced epithelial changes may reduce the corneal epithelial resistance to microbial invasion (10). An understanding of factors that contribute to the adherence of *Acanthamoeba* organisms to corneal epithelium may therefore be useful in understanding the pathogenesis of amebic keratitis. Recent investigations of the initial adherence of such ocular pathogens as *P. aeruginosa*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* to the host corneal epithelial cell have been performed by using organ or cell cultures (20, 21, 23). Coincubation assays based on morphologic examination of *Acanthamoeba* strains adherent to corneal cups or monolayers of SIRC cells have been described but are relatively subjective and somewhat inconsistent (14, 16). This is the first report of a quantitative in vitro model of adherence of radiolabeled *Acanthamoeba* strains to mammalian corneal epithelium.

Deposits consisting of a mixture of proteins, carbohydrates, lipids, and minerals build up rapidly on contact lenses when they are worn, and these may not be completely removed by cleaning (8, 12). *P. aeruginosa* organisms adhere in far greater numbers to worn lenses than to new, unworn lenses, suggesting that these deposits may serve as attachment sites for organisms already contaminating storage cases (3). The deposits are derived partially from corneal epithelial cell debris and ocular mucin and contain many of the same components as the corneal epithelium. Rapid adherence of *Acanthamoeba* organisms to corneal epithelium at 25°C suggests that if deposits serve as attachment sites for ameba, even short periods of exposure of worn contact lenses to contaminated lens solutions would be adequate for ameba to firmly attach.

It has been suggested that strains of *Acanthamoeba* which cannot grow axenically at 37°C have little chance of being virulent (5). Since the temperature of the cornea is only 34.3 ± 0.7°C, amebic strains which encyst at 37°C and do not experimentally induce encephalitis could still potentially cause keratitis (7). Under the experimental conditions described here, adherence of *Acanthamoeba* organisms to corneal epithelium was optimal at 25°C; at temperature extremes (4 and 37°C), trophozoites appeared to initially attach and then detach, possibly undergoing encystment. Nonetheless, significant adherence occurred at 37°C in just 20 min. In a multilayered organ culture system, or in vivo, the trophozoites might be able to quickly establish themselves in the corneal epithelium by migrating between and under the corneal cells before undergoing encystment. The temperature range over which adherence can successfully continue without encystment and what role, if any, amebic culture conditions play in influencing amebic adherence to the corneal epithelium are as yet undetermined. We chose to use organisms which were grown at room temperature, since most cases of amebic keratitis probably arise from organisms which initially grew in contact lens solutions kept at room temperature.

Different strains of *Acanthamoeba* differed significantly from each other in their ability to adhere to corneal epithelium, but these differences did not consistently correlate with the strains' pathogenicity as determined by mouse inoculation and by isolation from human patients. The end point of the mouse inoculation assay is death due to encephalitis, so it is possible that this is not a valid means of assessing potential pathogenicity of amebic strains for the cornea; i.e., adherence could still be a virulence determinant for amebic strains which cause keratitis. The fact that the strains which were identified as nonpathogens were environmental isolates with genotypes different from those of strains isolated from clinical cases of keratitis supports the assumption that these strains are nonpathogenic for the cornea.

Acanthamoeba adherence to the corneal epithelium was inhibited only by mannose compounds; none of the other sugars reduced adherence. Mannose has also been identified as a receptor for *P. aeruginosa* as well as *Candida albicans*, both of which are documented agents of contact lens-associated keratitis (6, 20). Contact lens-induced alterations in epithelial mannose moieties may increase corneal susceptibility to adherence and invasion by *Acanthamoeba* and other contact lens-associated pathogens. Corneal epithelium beneath a contact lens binds greater amounts of concanavalin A, wheat germ agglutinin, and *Maclura pomifera* agglutinin lectins than does exposed cornea (13). Similar changes are seen in corneal epithelium migrating to cover a wound, indicating that such glycoprotein or glycolipid alterations may provide a connecting link between keratitis

developing secondarily to ocular trauma and keratitis associated with contact lens wear (25).

Utilization of the mannose specificity of *Acanthamoeba* strains could lead to identification of an amebic adhesin, such as the galactose-specific lectin of *E. histolytica* (19). This lectin, although equally present in pathogenic and nonpathogenic strains, appears to be antigenically different in noninvasive strains (18). It is hoped that this antigenic difference can be exploited to identify those patients with entamoebiasis who are at risk for developing invasive disease. A number of glycoconjugates on the surface of *Acanthamoeba* organisms have been identified, but none are yet recognized as adhesins (17).

It is hoped that the use or adaptation of this adherence assay will provide additional information regarding mechanisms of adhesion of *Acanthamoeba* strains to corneal epithelium or other cells of corneal origin (e.g., stromal fibroblasts). The assay may also prove useful in screening potential therapeutic or prophylactic compounds aimed at inhibiting amebic adherence. Since this is a coincubation assay, toxicity of the compound to the corneal epithelium could be simultaneously assessed. This is an especially important consideration for antiprotozoal agents, which often have toxic side effects for mammalian cells.

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